MAJOR ARTICLE

Risk of Acquired Drug Resistance during Short-Course Directly Observed Treatment of Tuberculosis in an Area with High Levels of Drug Resistance

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Background. Data on the performance of standardized short-course directly observed treatment (DOTS) of tuberculosis (TB) in areas with high levels of drug resistance and on the potential impact of DOTS on amplification of resistance are limited. Therefore, we analyzed treatment results from a cross-sectional sample of patients with TB enrolled in a DOTS program in an area with high levels of drug resistance in Uzbekistan and Turkmenistan in Central Asia.

Methods. Sputum samples for testing for susceptibility to 5 first-line drugs and for molecular typing were obtained from patients starting treatment in 8 districts. Patients with sputum smear results positive for TB at the end of the intensive phase of treatment and/or at 2 months into the continuation phase were tested again.

Results. Among 382 patients with diagnoses of TB, 62 did not respond well to treatment and were found to be infected with an identical *Mycobacterium tuberculosis* strain when tested again; 19 of these patients had strains that developed new or additional drug resistance. Amplification occurred in only 1.2% of patients with initially susceptible or monoresistant TB strains, but it occurred in 17% of those with polyresistant strains (but not multidrug-resistant strains, defined as strains with resistance to at least isoniazid and rifampicin) and in 7% of those with multidrug-resistant strains at diagnosis. Overall, 3.5% of the patients not initially infected with multidrug-resistant TB strains developed such strains during treatment. Amplification of resistance, however, was found only in polyresistant Beijing genotype strains.

Conclusions. High levels of amplification of drug resistance demonstrated under well-established DOTS program conditions reinforce the need for implementation of DOTS-Plus for multidrug-resistant TB in areas with high levels of drug resistance. The strong association of Beijing genotype and amplification in situations of preexisting resistance is striking and may underlie the strong association between this genotype and drug resistance.

Resistance to tuberculosis (TB) drugs is emerging as a threat to control of TB in many areas, particularly in countries of the former Soviet Union [1]. High levels of multidrug-resistant (MDR) TB, defined as TB with resistance to at least isoniazid and rifampicin (the 2 most powerful TB drugs currently available), are consistently demonstrated in surveys conducted in the region [1–4]. Despite this finding, access to drug susceptibility testing and to appropriate second-line drug therapy for MDR TB is limited [5, 6]. As a result, most patients are treated with standard first-line chemotherapy, such as that recommended by the short-course directly observed treatment (DOTS) strategy [5, 6]. Not surprisingly, treatment of MDR TB with standardized short-course chemotherapy results in substantially poorer treatment outcomes than does treatment of drug-susceptible TB strains [7]. In addition, standardized treatment of patients infected with strains already

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resistant to some first-line drugs promotes the risk of the development of additional drug resistance caused by inadvertent inadequate therapy.

Resistance to TB drugs arises from inadequate chemotherapy because of either inappropriate prescription, poor drug quality or supply, or poor adherence to treatment [1]. These conditions can result in the effective exposure of bacilli to a single drug; under these conditions, small numbers of spontaneously occurring drug-resistant mutants have a selective advantage and, therefore, multiply [8]. For this reason, it is well known that a single drug should not be added to a failing regimen. A regimen that includes drugs against which a strain has primary drug resistance may have the same effect as single-drug therapy in terms of the selection of resistant subpopulations. This effect has been termed "amplification" of drug resistance [9] and has been demonstrated in several contexts [10–12].

To quantify the extent of acquired drug resistance during DOTS chemotherapy in an area with a high level of initial drug resistance, we assessed data from a drug resistance surveillance study conducted in 2 regions of Central Asia: Karakalpakstan in Uzbekistan and Dashoguz in Turkmenistan (rates of MDR TB in 2001 were 27% in Karakalpakstan and 11% in Dashoguz) (Appendix; online only).

METHODS

Study design. This study is an extension of a cross-sectional drug resistance survey conducted in both Karakalpakstan and Dashoguz. Patients were recruited as previously described [2]. Additional details are described in the Appendix (online only).

TB treatment and additional sampling. In addition to sputum samples obtained at diagnosis and used for the drug resistance survey, sputum samples were obtained when a patient continued to have positive sputum smear results at the end of the intensive phase of treatment and/or at 2 months into the continuation phase of treatment. At the time of the survey, there was no capacity to treat drug-resistant cases of TB using DOTS-Plus for MDR TB in either region. For this reason, patients were placed on standard DOTS regimens (category I or II) in accordance with their prior TB treatment history, irrespective of drug resistance results. New patients who had not previously received treatment for TB for $\geqslant$1 month were given a category I regimen consisting of daily doses of isoniazid, rifampicin, pyrazinamide, and ethambutol, with or without streptomycin for 2 months, followed by isoniazid and rifampicin 3 times weekly for 4 months. The category II regimen for patients previously treated for TB for $\geqslant$1 month consisted of receipt of all 5 drugs daily for 3 months minus streptomycin for the last month, followed by isoniazid, rifampicin, and ethambutol 3 times weekly for 5 months. Patients were hospitalized during the intensive phase of treatment and received doses during the continuation phase that were ostensibly administered under direct observation by local health care workers. Although information on adherence to treatment (doses observed, doses given without observation, and missed doses) was routinely recorded, the data were not considered to be accurate enough to use for analysis.

Laboratory testing and statistical analysis. Because there were no facilities for culture of sputum samples available in either region or country at the time of the survey, sputum samples were transported directly from both Karakalpakstan and Dashoguz to the Supranational Reference Laboratory in Borstel, Germany. Primary isolation and culture of mycobacterial isolates were performed as described elsewhere [13]. All strains were tested for susceptibility to the 5 first-line drugs used in the DOTS program on Löwenstein-Jensen media, using the proportion method. If there was insufficient growth, drug susceptibility was tested using the modified proportion method in Bactec 460TB (Becton Dickinson Microbiology Systems).

Extraction of genomic DNA from mycobacterial strains and DNA fingerprinting using IS*6110* as a probe were performed according to a standardized protocol [14]. In addition, all isolates were analyzed by the spoligotyping technique [15]. Molecular typing data were analyzed with Bionumerics software (Windows NT, version 3.5; Applied Maths). The spoligotyping data were used to additionally confirm strain relationships and to identify Beijing genotype isolates (no hybridization to spacers 1–34, but hybridization to spacers 35–43). EpiInfo software, version 6.04d (Centers for Disease Control and Prevention), was used to calculate 95% CIs for proportions by Fisher's exact test and to compare proportions by χ^2 test.

RESULTS

Study population and additional testing. In total, 416 patients were included in the original drug resistance survey (213 patients were from Karakalpakstan, and 203 patients were from Dashoguz). Of these patients, 397 had strain cultures available for IS*6110* DNA fingerprinting and spoligotyping analysis (DNA testing). The 19 strains from the remaining patients did not grow at the time of DNA testing. Of these 397 strains, 382 demonstrated clear-cut IS*6110* banding patterns, and 15 showed mixed banding patterns, demonstrating double infection with 2 *Mycobacterium tuberculosis* strains. The 382 patients with identifiable isolates form the population for this study. These 382 patients were not different from the full sample included in the drug resistance survey with regard to age, sex, district of residence, or outcome of DOTS. The numbers of new and previously treated patients, by region and by categories of drug resistance and success of DOTS, are shown in table 1.

For analysis of amplification of resistance, the 2 regions were combined according to similar treatment outcomes, given the same level of drug resistance and because of the small number of patients in each resistance category. Overall, additional spu-

Table 1. Drug resistance and success of the short-course directly observed treatment (DOTS) regimen among new and previously treated patients with tuberculosis in Karakalpastan, Uzbekistan, and Dashoguz, Turkmenistan.

	Karakalpakstan (n = 198)		Dashoguz (n = 184)	
Drug resistance category	New patient (n = 109)	Previously treated patient (n = 89)	New patient (n = 100)	Previously treated patient (n = 84)
Pansusceptible	52 (79)	21 (62)	71 (82)	33 (67)
Monoresistant	20 (80)	14 (59)	20 (100)	18 (61)
Polyresistant	20 (55)	21 (67)	5 (100)	19 (58)
MDR[a]	17 (24)	33 (24)	4 (50)	14 (21)

NOTE. Data are no. of patients (% of patients experiencing success of DOTS regimen). MDR, multidrug resistant.

[a] Resistant to at least isoniazid and rifampicin.

tum samples were obtained from 82 patients during treatment. Of these patients, 62 were found to be infected with the same strain of *M. tuberculosis* as that identified at diagnosis on the basis of identical DNA fingerprinting of the second samples. The remaining 20 patients were excluded on the basis of a different strain found at retesting (10 patients), double infection at retesting (5 patients), and possible laboratory contamination (5 patients). Of the 62 patients, 34 had paired identical isolates at diagnosis and at the end of the intensive phase of treatment, and 14 had paired identical isolates at diagnosis and at 2 months into the continuation phase of treatment. The remaining 14 patients were found to have identical isolates at both the end of the intensive phase and at 2 months into the continuation phase.

Amplification of drug resistance. The drug resistance profiles of the 382 patients at diagnosis revealed high levels of preexisting drug resistance; >50% of patients were infected with a strain showing some level of drug resistance, and approximately one-third were infected with strains resistant to ⩾2 first-line drugs (table 2). Overall, 19 (31%) of the 62 strains from patients infected with identical strains at second testing developed new or additional drug resistance during treatment (table 2). All but 2 of these patients were infected with initially drug-resistant strains at diagnosis. In addition to the 2 instances of the development of drug resistance by initially pansusceptible strains, 1 strain that was monoresistant at diagnosis acquired additional drug resistance, giving an overall rate of amplification of 1.2% (95% CI, 0.3%–3.5%) for these groups. In contrast, 17% of strains with preexisting resistance to >1 drug but not isoniazid and rifampicin, defined here as polydrug resistance, amplified their resistance during short-course chemotherapy (table 2). All except 1 of these strains developed additional rifampicin resistance to become MDR. Significant amplification was also found among the strains found to be MDR at diagnosis, with 7% (95% CI, 2%–16%) developing additional first-line resistance (table 2).

Drug resistance at diagnosis and resistance amplified during treatment are shown in table 3. Nineteen patients had strains that amplified their resistance; 11 strains became MDR during DOTS, and 12 developed either isoniazid or rifampicin resistance. Overall, MDR TB strains developed during treatment in 11 of 314 patients who were not already infected with MDR TB strains at diagnosis (3.5%; 95% CI, 1.8%–6.2%). Among the strains that were already MDR, pyrazinamide was the most common drug to which strains developed additional resistance. Most of the measured amplification of resistance occurred during the intensive phase of treatment, when patients were taking the maximum number of drugs and were hospitalized. Ten of the 19 patients were new patients and were therefore undergoing category I DOTS.

A striking observation was that resistance to both isoniazid and streptomycin at diagnosis posed a significant amplification risk, with 5 (12%) of 41 patients developing MDR TB strains during treatment. This was not the case among the 21 patients with isoniazid-monoresistant strains (table 2). An additional interesting finding was that all 5 isoniazid- and streptomycin-resistant TB strains that amplified their resistance were found to be of the Beijing genotype. Among all polyresistant strains, 11 (39%) of 28 polyresistant Beijing genotype strains amplified their resistance, compared with none of the 27 non-Beijing polyresistant strains ($P < .05$).

Among the 19 patients with strains that amplified their resistance, treatment failure was the most common outcome of DOTS treatment (in 13 patients). Of the remaining patients, 3 died during treatment, 1 defaulted (while continuing to have positive sputum smear test results), and 2 were recorded as being successfully treated after the intensive phase of treatment was extended. However, 1 of the successfully treated patients subsequently developed disease that was diagnosed by a positive sputum smear test result.

Changes from resistant to susceptible. There were some instances of strains that were initially resistant becoming sus-

Table 2. Number of strains at diagnosis, at retesting, and demonstrating amplification of resistance during treatment, by drug resistance categories at diagnosis.

First-line drug resistance profile at diagnosis	No. of strains at diagnosis	No. (%) of identical strains at retesting[a]	Amplification detected among identical strains, no. of strains (% of strains at diagnosis; 95% CI)
Pansusceptible	177	12 (7)	2 (1.1; 0.1–4.0)
Monoresistant			
All	72	10 (14)	1 (1.4; 0.0–7.5)
Isoniazid	21	1 (5)	0
Rifampicin	1	0	0
Ethambutol	0	0	0
Streptomycin	49	9 (18)	1 (2)
Pyrazinamide	1	0	0
Polyresistant			
All	65	16 (25)	11 (17; 9–28)
Isoniazid, streptomycin	41	9 (22)	5 (12)
Isoniazid, pyrazinamide	3	0	0
Isoniazid, ethambutol	1	0	0
Isoniazid, ethambutol, streptomycin	12	4 (33)	3 (25)
Isoniazid, streptomycin, pyrazinamide	6	2 (33)	2 (33)
Isoniazid, ethambutol, streptomycin, pyrazinamide	2	1 (50)	1 (50)
MDR[b]			
All	68	24 (35)	5 (7; 2–16)
Isoniazid, rifampicin	1	1 (100)	1 (100)
Isoniazid, rifampicin, streptomycin	19	5 (26)	2 (11)
Isoniazid, rifampicin, streptomycin, pyrazinamide	3	2 (67)	0
Isoniazid, rifampicin, ethambutol, streptomycin	30	12 (40)	2 (7)
Isoniazid, rifampicin, ethambutol, streptomycin, pyrazinamide	15	4 (27)	0
Overall	382	62 (16)	19 (5; 3–8)

NOTE. MDR, multidrug resistant.

[a] Only patients who had positive sputum smear results for tuberculosis during treatment were retested for drug resistance.

[b] Resistant to at least isoniazid and rifampicin.

ceptible to drugs. Five strains lost resistance to ethambutol and 3 strains lost resistance to streptomycin over the course of treatment. All of the strains initially measured as ethambutol resistant and later as ethambutol susceptible were also resistant to several other drugs.

DISCUSSION

Our study, conducted in an area with high levels of drug resistance, demonstrates the high levels of amplification of drug resistance that occur when patients are treated with standard first-line regimens in a DOTS program. Patients infected with polyresistant TB strains are at risk of developing MDR TB when treated with standard short-course chemotherapy. Overall, 17% of TB strains from these patients amplified their drug resistance, whereas 7% of strains that were already MDR also developed additional first-line drug resistance. In total, 3.5% of patients not initially infected with MDR TB strains were found to have developed MDR strains during treatment. The majority of patients whose strains demonstrated amplification of resistance also experienced poor outcomes of treatment, with treatment failure or death predominating.

Our knowledge about the performance of standardized DOTS in areas with high levels of drug resistance and the potential impact of DOTS on amplification of resistance is limited. A large study performed in the Tomsk region of Russia, where ~20% of all patients with TB have MDR TB strains [1], revealed results similar to those presented here. In Tomsk, failure of a DOTS category I treatment regimen was strongly associated with developing drug resistance. Acquired MDR TB was demonstrated in 55% of patients who were not initially infected with MDR strains and who experienced treatment failure [12]. Similar to our study, the highest rate of amplification of resistance was recorded among patients with preexisting isoniazid or rifampicin resistance but not both; among this group,

Table 3. Amplification of drug resistance during treatment and treatment outcomes for 19 patients with tuberculosis in whom amplification was detected.

Treatment category, patient	Beijing genotype	Drug resistance at diagnosis	Resistance at end of intensive phase	Resistance 2 months into continuation phase	Treatment outcome	Drug resistance amplified during treatment
Previously untreated patient[a]						
1	No	Susceptible	S	S	Failure	S
2	Yes	H, S	…	H, R, S	Failure	R
3	Yes	H, S	…	H, R, S	Failure	R
4	Yes	H, S	H, R, S	H, R, S	Failure	R
5	Yes	H, S	H, R, S	…	Default	R
6	Yes	H, E, S	H, R, E, S	H, R, S	Failure	R
7	Yes	H, S, Z	H, E, S, Z	…	Completion	E
8	Yes	H, R, S	H, R, E, S, Z	…	Failure	E, Z
9	Yes	H, R, S	H, R, S, Z	…	Death	Z
10	Yes	H, R, E, S	H, R, E, S, Z	…	Cure	Z
Previously treated patient[b]						
11	No	Susceptible	Susceptible	H, R, S	Failure	H, R, S
12	No	S	H, S	…	Failure	H
13	Yes	H, S	H, R, E, S	…	Failure	R, E
14	Yes	H, S, Z	H, R, E, S, Z	…	Death	R, E
15	Yes	H, E, S	H, R, E, S	H, R, E, S	Failure	R
16	Yes	H, E, S	H, R, E, S, Z	…	Failure	R, Z
17	Yes	H, E, S, Z	H, E, S, Z	H, R, S, Z	Failure	R
18	No	H, R	…	H, R, E, S	Failure	E, S
19	Yes	H, R, E, S	H, R, E, S, Z	H, R, Z, S	Death	Z

NOTE. Susceptible is defined as pansusceptible to all 5 first-line drugs tested. E, ethambutol; H, isoniazid; R, rifampicin; S, streptomycin; Z, pyrazinamide.

[a] New patients (previously untreated) received the following category I regimen: daily H, R, Z, and E, with or without S for 2 months, followed by H and R 3 times weekly for 4 months.

[b] Patients previously treated received the following category II regimen: H, R, Z, E, and S daily for 3 months minus S for the last month, followed by H, R, and E 3 times weekly for 5 months.

71% developed MDR TB strains. Alarmingly, 18 of 31 patients experiencing treatment failure in Tomsk who were infected with drug-susceptible or streptomycin-monoresistant TB strains at diagnosis also developed drug-resistant TB, most often MDR TB.

However, the Tomsk study [12] did not include molecular typing (DNA fingerprinting) of additional culture specimens obtained from patients showing the development of resistant TB strains and, thus, could not distinguish between the various reasons for changes in drug resistance profiles during treatment, such as amplification of resistance, mixed infection at diagnosis, or reinfection with a second strain. It has been demonstrated that a significant proportion of patients can be infected with multiple strains of *M. tuberculosis* [16–18]. Given this scenario, a previously undetected drug-resistant strain might emerge under the pressure of chemotherapy as drug-susceptible strains are killed. Multiple infections may also arise through the superinfection of patients receiving treatment [19]. Furthermore, it has been demonstrated that exogenous reinfection with a second resistant or even MDR TB strain can be a significant cause of treatment failure and of the emergence of resistance [20–22]. Finally, without DNA fingerprinting, laboratory contamination cannot be ruled out [23].

In contrast, our study included molecular genotyping of strains obtained from a second set of samples, which allowed likely reinfections, double infections, and potential laboratory contaminations to be excluded. Only 3 instances of the acquisition of drug resistance among initially pansusceptible or monoresistant strains were found in this study (1.2% of this group). In 1 of these cases, isoniazid, rifampicin, and streptomycin resistance developed during the ambulatory, continuation phase of treatment, when drug taking is not as well supervised as it is during the hospitalized, intensive phase. The development of streptomycin resistance may well be a result of the practice of continuing streptomycin treatment privately during DOTS on the basis of the common view that injections are better than tablets [24]. Poor adherence can lead to drug resistance in initially drug-susceptible strains because of intermittent periods of not taking drugs or because of selectively taking some drugs [8]. The relatively low level of acquisition of drug resistance among pansusceptible or monoresistant strains is reassuring and suggests that poor adherence is not a

significant factor in the creation of drug resistance in the DOTS program.

The highest rate of amplification of resistance was observed among isoniazid- and streptomycin-resistant isolates, whereas none of the isoniazid-monoresistant strains developed further resistance. This is somewhat surprising, considering that 3 drugs (rifampicin, pyrazinamide, and ethambutol) in the standard regimen would be expected to be active both for patients with combined isoniazid and streptomycin resistance and for those with isoniazid monoresistance. However, our data are in accordance with the results of a study performed in Vietnam, in which combined resistance to isoniazid and streptomycin was found to be a strong risk factor for treatment failure and relapse of TB and for acquired multidrug resistance among patients experiencing treatment failure and relapse of TB [10].

The availability of mycobacterial genotyping data also allowed us to investigate the association between infection with Beijing genotype strains and amplification of resistance. Although the number of patients was small, amplification of resistance appeared to be more common among strains of the Beijing genotype; nearly one-third of polyresistant Beijing strains amplified their resistance during standard DOTS chemotherapy, whereas none of the non-Beijing strains with a similar resistance profile did so.

These data indicate that, in cases of preexisting polyresistance, Beijing genotype strains have a higher capacity to develop further drug resistance than do strains of other *M. tuberculosis* complex genotypes. Such a capacity might then result in a selective advantage for Beijing genotype strains in regions with high levels of drug resistance. This is in accordance with and might be the reason for the high rates of Beijing genotype strains found in several regions of the former Soviet Union and for the observed association with MDR TB [25–27]. The mechanism potentially allowing Beijing genotype strains to develop resistance more readily is not known. Our results indicate that, especially for Beijing genotype strains, alternative treatment regimens might be required to avoid amplification of resistance and development of MDR TB. Therefore, further research is urgently needed to determine the implications of these findings for treatment of individual patients in areas with high levels of drug resistance.

The levels of amplification of drug resistance found in our study are likely to be a significant underestimate of the true situation. Only patients who had positive sputum smear results during treatment were retested for drug resistance. Because sputum smear microscopy is relatively insensitive, particularly during treatment, it is possible that more patients could have remained culture-positive for TB throughout treatment [28]. Had sputum samples from all patients been cultured, additional resistance amplification might have been detected. Indeed, many of the patients infected with drug-resistant strains of TB at diagnosis who were classified as successfully treated with DOTS were subsequently found to have smear-positive TB [29].

There is a range of possible explanations for the changes in drug resistance from resistant to susceptible that were observed in some cases in our study. First, proficiency testing suggests that drug susceptibility testing is less accurate for ethambutol and streptomycin than it is for rifampicin and isoniazid [30]. Drug susceptibility testing in our study was conducted by the National Reference Centre for Mycobacteria in Germany, a member of the Supranational Reference Laboratory Network established in 1994 [31]. As a member of the Supranational Reference Laboratory Network, our laboratory participates in a quality assurance and proficiency testing program. Testing for susceptibility to ethambutol is often problematic; there is a small difference between the critical concentration used for drug susceptibility testing and the MIC, which may explain the discordant results [32, 33]. For streptomycin, similarly discordant results may be explained by observations from some of the early trials of streptomycin treatment in which reversion of streptomycin resistance to susceptibility was shown to occur [34]. Finally, it has previously been demonstrated that bacillary populations from the same patient can display different resistance profiles [35–37]. Different resistance profiles in sputum samples obtained throughout treatment may, therefore, represent different bacillary populations in different parts of the lung.

In conclusion, our study demonstrated a high risk of amplification of drug resistance among patients treated using standard DOTS regimens. These results indicate that, in areas with high levels of drug resistance in which >40% of all smear-positive patients are infected with strains of TB with initial first-line drug resistance, systematic drug susceptibility testing, followed by appropriate treatment for drug-resistant TB, is required to identify those at high risk for both treatment failure and amplification of drug resistance and, therefore, subsequent death. The additional laboratory costs associated with culture and drug susceptibility testing need to be weighed against the cost of treating newly created additional cases of MDR TB with lengthy second-line drug regimens. Additional work is required to determine the level of drug resistance among patients with TB (both new and previously treated) at which routine drug susceptibility testing should be used to avoid the creation of unnecessary and costly drug resistance.

Furthermore, our data indicate that strains of the Beijing genotype with preexisting resistance have a higher risk of developing additional resistance during standard treatment. This has potentially serious consequences for the diagnosis and treatment of TB and suggests that further longitudinal studies in areas with a high incidence of TB are required to investigate this phenomenon and confirm our findings.

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